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PRINCIPAL INVESTIGATOR: Jonathan Rosenberg, M.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

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Jonathan Rosenberg, M.D.

E-Mail: jrosenbe@medicine.ucsf.edu

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14. ABSTRACT

This annual report for the Physician Research Training Award focuses on progress in the genetic analysis of circulating hormone refractory prostate cancer micrometastases. As metastatic tissue is often inaccessible in advanced prostate cancer patients, analysis of circulating tumor cells may provide understanding of the biology of hormone refractory prostate cancer as well as chemotherapy resistance. Oligonucleotide array comparative genomic hybridization allows the assessment of genetic changes that may occur in the process of metastasis and chemotherapy resistance. Genomic profiling using this technology will go beyond cell counting, and circumvent technical complexities related to working with RNA. Work performed over the last year has perfected techniques to deal with small amounts of DNA isolated using the Vitatex cell isolation system. Preliminary data suggests that reproducible genomic alterations are observed in the circulating tumor cells isolated from patients with metastatic hormone refractory prostate cancer. During year 3, having ironed out the methodology of pursuing this work, we will ramp up collection of peripheral blood on patients with chemotherapy-naïve and chemotherapy-refractory hormone refractory prostate cancer to isolate circulating tumor cells and perform genetic analyses.

15. SUBJECT TERMS
circulating tumor cells, prostate cancer, chemotherapy resistance, genomic analysis

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Introduction

This project focuses on the genetic analysis of circulating hormone refractory prostate cancer micrometastases focusing on the mechanisms of chemotherapy resistance. Metastatic tissue for research is difficult to obtain in hormone refractory prostate cancer (HRPC), as most metastatic sites are not conducive to biopsy. However, circulating tumor cells (CTC's) have been found in high numbers in patients with metastatic HRPC. CTC's represent an untapped resource for studying metastatic prostate cancer. These cells are easily accessible in the peripheral blood. The purpose of this research is to detect genetic alterations that occur during the development of chemotherapy resistance, to give insight into the mechanisms behind this resistance, and determine potential therapeutic strategies to combat it. We have refined the techniques needed to isolate the CTCs, isolate genomic DNA from those cells, amplify the DNA if necessary, and evaluate genomic alterations using oligonucleotide comparative genomic hybridization. Evaluation of the genomic alterations in chemotherapy naïve and chemotherapy refractory HRPC CTC's is ongoing. Preliminary results suggest that CTCs isolated from different patients using the Vitatex technology contain recurrent genomic alterations. Several grant proposals have been submitted to secure outside funding for this research. This report will summarize the progress on this grant and the challenges and obstacles that have arisen and how they will be overcome.

Tasks 1, 2: *Isolation and characterization of circulating micrometastases of chemotherapy naïve and chemotherapy resistant HRPC.*

Over the last year, procedures and techniques were devised and revised to allow for the capture of cells using the CAM-attached cells at UCSF. This has taken a significant amount of effort and time. The methodology of DNA amplification for CTC DNA was piloted and preliminary data shows it should work with good fidelity compared with unamplified DNA. In addition, initial experiments evaluating genomic changes in CTCs from HRPC patients show promising results, described below.

Most prior studies involving CTCs in prostate cancer patients have been enumeration studies or expression studies. Expression is dependent on RNA extraction procedures and on environment. Therefore, the disparate published results may be because RNA specimen collection is very finicky and expression is largely dependent on the environment. Expression profiles of CTCs may share only limited concordance with cells from the primary tumor and significant variation within and between patients is expected. Genomic profiling will go beyond cell counting, and circumvent technical complexities related to working with RNA.

We have been able to extract approximately 10 micrograms of DNA from isolated cells taken from 20 mL of peripheral blood for use in genomic analysis. This year, the clinical trial, "A Phase I/II Study of Docetaxel/ Prednisone and PTK787/ZK222584 in Previously Untreated Metastatic Hormone Refractory Prostate Cancer," closed for lack of accrual. Therefore, we will be collecting specimens from chemotherapy naïve patients on another phase II study, "A Randomized Phase II Study of Intermittent Chemotherapy or Intermittent Chemotherapy with Maintenance GM-CSF in Patients with Previously Untreated Metastatic Hormone Refractory Prostate Cancer," as well as from other taxane-naïve HRPC patients. In addition, accrual to the chemotherapy-refractory hormone refractory prostate cancer trial, UCSF 055513, "Phase I/II Trial of Epothilone Analog BMS-247550 (Ixabepilone), Mitoxantrone, and Prednisone in Hormone Refractory Prostate Cancer Patients Previously Treated with Chemotherapy," has proceeded more slowly than expected, and only 6 patients at UCSF have been enrolled to date. A total of 7 patients have been collected to date, and we have been able to isolate CTC's from 6 of these specimens. Now that technical details have been worked out and we have preliminary results (see below) suggesting that we are able to isolate CTCs using this technology, we will now increase accrual over the next year. I plan to collect approximately 30 specimens, roughly evenly divided between chemotherapy-naïve and chemotherapy refractory HRPC. In addition, we will be piloting another technology with collaborators at CalTech using a size selective filter for isolation of CTCs.

Tasks 3,4,5,6
Analyze and compare gene signatures of circulating tumor cells to biomarkers previously identified, identify markers of chemotherapy resistance and response in CTC's in HRPC.

Currently oligonucleotide comparative genomic hybridization (oCGH) requires 500ng of input DNA. However, the amount of DNA isolated from circulating tumor cells may be less than 500ng. Because the same issue confronts clinical application of array CGH, the

Paris/Collins laboratory has been evaluating linear and rolling circle methods for the isolation of DNA from formalin-fixed paraffin-embedded (FFPE) biopsy specimens (manuscript in preparation). Data suggests that it is possible to obtain DNA from paraffin that works very well for array and oligonucleotide CGH and that whole genome amplification (WGA) does not introduce unacceptable copy number artifacts as determined using array CGH. Similar oCGH profiles obtained with unamplified and matching WGA amplified FFPE prostate DNA. Therefore, if necessary, extraction of DNA from circulating tumor cells followed by whole genome amplification should provide sufficient high quality DNA for use with oCGH. FFPE biopsy samples can be treated similarly, if needed. These methodologies were refined over the last year and are now able to be applied to DNA isolated from CTCs.

An initial Vitatex evaluation was carried out by performing oligonucleotide CGH using DNA extracted from a single patient's CTCs isolated with the CAM technology (Figure 1). Our standard reference, commercial normal DNA obtained from blood, served as reference DNA during the hybridization for oCGH using a BAC array. The Paris/Collins lab has recently switched to the Agilent oCGH platform because it offers comparable data to the BAC arrays, but at a much higher resolution (35kb versus 1.4Mb) and works well with smaller amounts of DNA (500ng). (manuscript submitted). We tested commercial male and female reference DNA using the Agilent oCGH platform to determine the amount of background noise (Figure 2). The comparison revealed minimal differences outside of the X and Y chromosomes, as expected. As a result, the Agilent oCGH platform will be utilized for the CGH studies.

Preliminary data has been generated from 6 samples for which DNA was able to be isolated from CTCs isolated. DNA was extracted from unbound cells using the Vitatex isolation system (white blood cells) in a prostate cancer patient, and compared to reference normal DNA (Figure 3). No significant difference was found for the WBC fraction compared to normal DNA. These data are presented in Figure 3. In Figure 4, DNA isolated from CTCs is compared to reference normal male DNA. DNA copy number changes are observed in the CTC's compared to normal DNA controls. This is in contrast to white blood cell fractions from each patient shown in Figure 3 which do not demonstrate DNA copy number changes. This suggests that the alterations observed in the CTC fraction represent specific changes associated with CTCs. Furthermore, recurrent alterations are being identified in different CTCs from different patients, suggesting that genes may be present at the identified loci that are involved in HPRC pathogenesis. Further specimens from both chemotherapy naïve- and chemotherapy-refractory HRPC patients will be collected over the next year so statistically meaningful conclusions may be reached. DNA isolation from primary tumor specimens is ongoing, and no data is available at this time.

To further support the identity of the isolated cells as CTCs, immunohistochemistry is being performed on the isolated cells. Cytospins will be prepared using a 50 ml aliquot of the CTCs and the unbound, white blood cell-(WBC) fraction. The cells will be stained for cytokeratin and CD45, using a hematoxylin counterstain to visualize the DNA of the cell nuclei. The epithelial origin of the cells will be determined by a positive cytokeratin

stain and a negative CD45 stain. The WBC slide will serve as a negative control which should stain positive for CD45 and negative for the cytokeratin. As a positive control in the IHC studies, cytospins of cell cultured Du145 cells (ATCC) will be prepared. Du145 cells should stain positive for cytokeratin. IHC staining and analysis will be carried out in the UCSF Comprehensive Cancer Center Tissue Core. Dr. Jeff Simko will review all IHC slides. Results on the 7 specimens collected are pending.

Prior biopsy and prostatectomy specimens will be requested from all patients who provide blood samples for CTC isolation. All FFPE primary tumors will undergo a detailed pathology review by Dr. Jeff Simko in the UCSF Tissue Core, with regions of greater than 80% tumor marked. These specimens will have DNA isolated for oCGH and comparison with CTC DNA.

Enumerating CTCs in the blood of patients with prostate cancer has not previously proven
prognostic. As part of the work on this grant evaluating the importance of CTC's in HRPC, Dr. Rosenberg has been working with Dr. John Park and Dr. Jorge Garcia to determine whether the numbers of circulating tumor cells carries prognostic significance in patients with metastatic HRPC. This research has been developed into a manuscript that is in press at the British Journal of Urology at this time.

The lack of quality CTC detection methods has impeded efforts to detect, quantify and characterize CTCs. The various methods for detecting CTCs in prostate cancer include RT-PCR(1, 2), immunohistochemistry (IHC)(3), fluorescent-activated cell sorting (FACS)(4) and magnetic-activated cell sorting (MACS)(5). A limitation of using a PCR-based detection method is that one can detect products from nonmalignant cells present in the circulation. The use of IHC has resulted in contradictory results due to inconsistencies with antibody specificity and the reliance on subjective interpretation. Limitations of FACS and MACS include the requirement for expensive equipment and a labor intensive procedure.

Collaboration has been established with Drs. Richard Cote and Ram Datar at USC, who along with Dr. Tai's laboratory at Caltech, have developed a size exclusion type device to isolate CTCs from blood. The diameter of CTCs varies from 10 to 35 μm, with about 80% being 15-30μm wide, while the leukocytes are 5-20 μm, with 80% of them being 6-12μm wide. Therefore, they developed a parylene-membrane-based filter microdevice with precisely defined pore sizes and have shown a distinct potential for target tumor cell isolation from small blood volumes. Their initial unpublished results show a recovery rate of >85% for capturing cultured prostate cancer cells from the LNCaP cell line spiked in 1 ml of neat, undiluted blood. They have also demonstrated the sensitivity of their device by spiking 4 tumor cells in 1ml of phosphate buffered saline solution and successfully recovering at least 3 cells in each of 5 repeat experiments. Unfortunately, collaboration with Dr. Lydia Sohn regarding the nanocytometer has not progressed significantly due to personnel difficulties in her laboratory.

The Vitatex technology, which utilizes cell adhesion matrix (CAM) coated test tubes, and the USC/Caltech device are not cost-prohibitive or labor intensive. We will combine IHC detection with these CAM-based and size-based isolation procedures. The proposed coupling of the technologies (Vitatex-CAM, USC/Caltech-filter, IHC, oCGH) will enable isolation and detailed cellular and genomic characterization of CTCs from prostate cancer patients. Initial work with the USC filter in year 3 will test several samples as pilot work for possible future funded projects.

Additional funding has been applied for from the Prostate Cancer Foundation and the USCF Research and Evaluation Council. Another grant proposal is being prepared for the UCSF Clinical and Translational Science Strategic Opportunities Support Center.

Task 7

Educational component

Due to clinical and research responsibilities, formal coursework was not done during year 2. However, Dr. Rosenberg meets regularly with Dr. Small to discuss research and clinical trial design, as well as with Dr. Paris to discuss progress on CTC isolation and characterization. In addition, Dr. Rosenberg attends the UCSF Experimental Therapeutics monthly conference led by Dr. Doug Hanahan to discuss animal models of therapeutics. Dr. Rosenberg also participates in the weekly Urologic Oncology conference, and bi-weekly Hematology-Oncology conference.

Figure 1

Figure 1: oCGH result for the CTC DNA (unamplified) using normal commercial DNA from blood as a reference. Blue bars to the left of each chromosome line represent deletions and to the right denote amplifications. This specimen demonstrates gains and

losses that are different than reference normal DNA and represent genomic alterations in the CTC fraction.

Figure 2

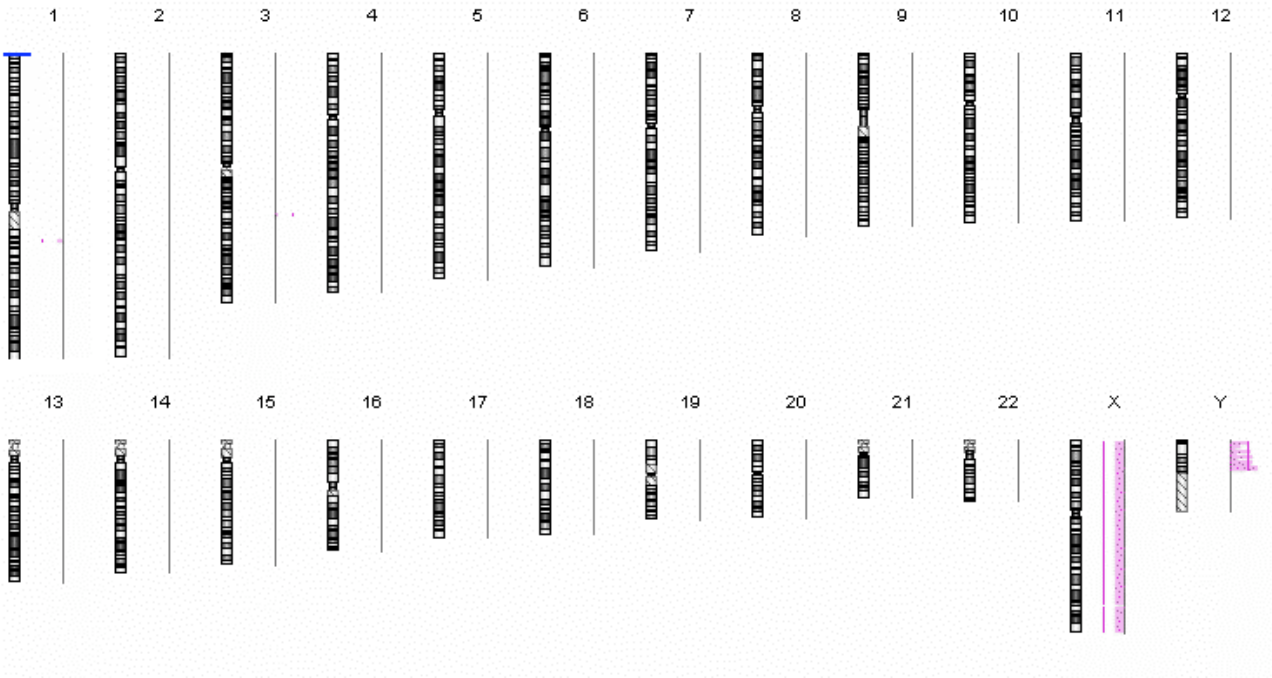

Figure 2 Hybridization of male versus female genomic DNA results in minimal random noise. Copy number calls are shown in pink. Next to each ideogram is a vertical line with losses shown to the left and gains to the right. The expected loss of X and gain of Y is observed.

Figure 3

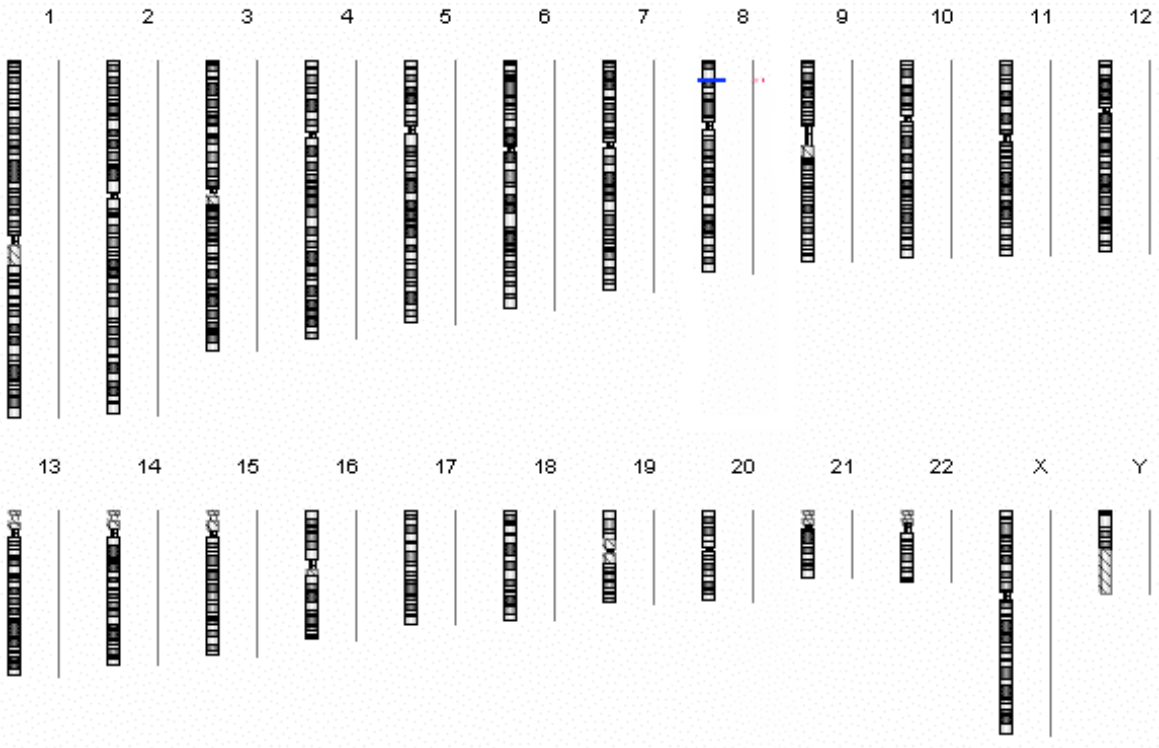

Figure 3. The unbound fraction, presumably white bloods cells, does not generally have copy number changes. This provides evidence that the changes we observe with the Vitatex technology for the bound fraction could be from circulating tumor cells.

Figure 4

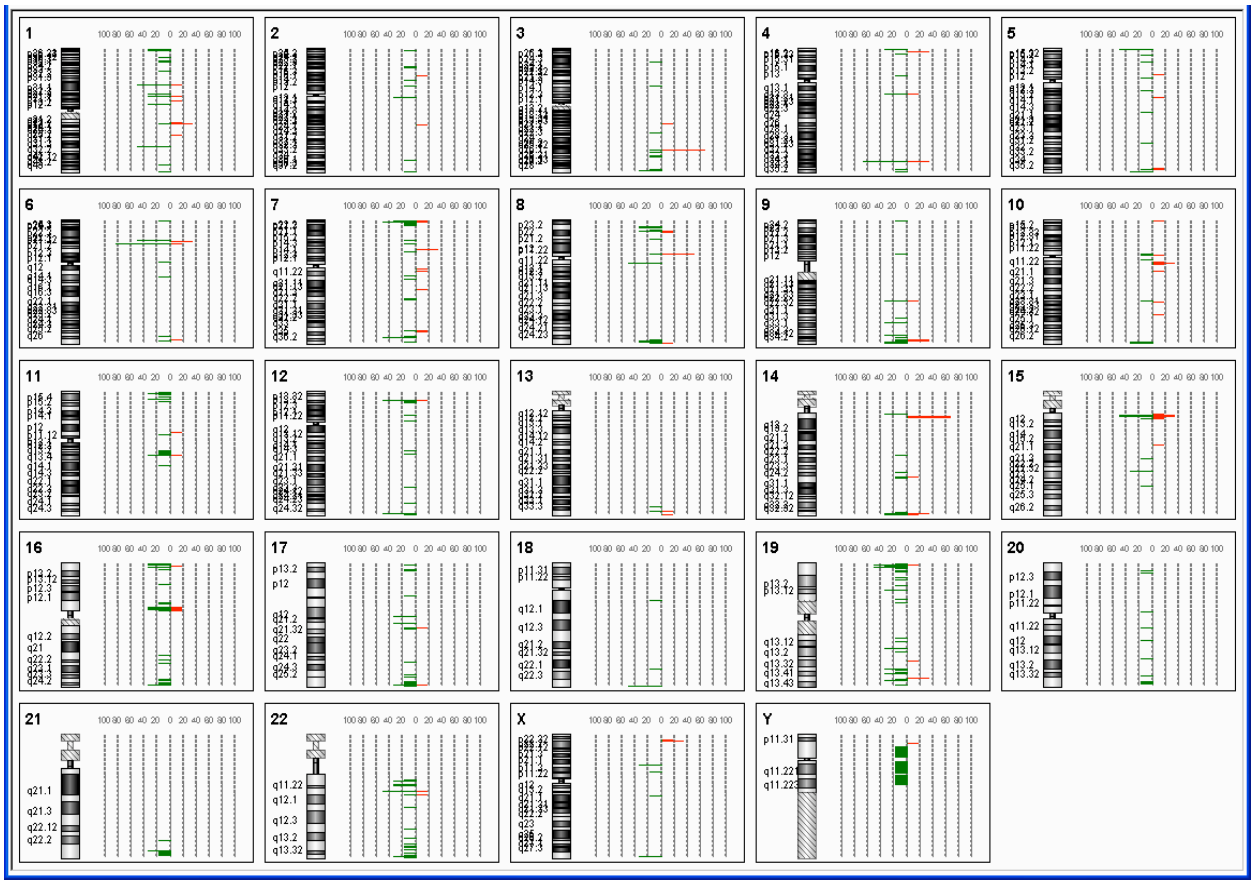

Figure 4. The frequency of copy number changes for the cohort is plotted against the chromosomal position. Gains are shown in red, losses in green. Recurrent changes are being observed.

Key Research Accomplishments:

- Identification of CTC's as predictive of survival in chemotherapy-naïve patients with HRPC treated with taxane-based chemotherapy
- Demonstration that reproducible genomic changes can be observed in CTCs using the Vitatex isolation technology, and that these changes are not observed in white blood cells collected from those patients.
- Identification of a collaborator at USC/CalTech to test another CTC isolation platform in HRPC.

Reportable Outcomes:

"Evaluation and Significance of Circulating Epithelial Cells in Hormone Refractory Prostate Cancer Patients"

[1]Jorge A. Garcia, [2] Jonathan E. Rosenberg, [1] Vivian Weinberg, [1] Janet Scott, [1] Mark Frohlich, [1] John Park, [1] and Eric J. Small [1, 3]
From the Departments of Medicine,[1] University of California, San Francisco, UCSF Comprehensive Cancer Center Biostatistics Core, [2] Department of Urology, [3] UCSF Comprehensive Cancer Center. In press, British Journal of Urology, January 2007.

"Activity of second-line chemotherapy in docetaxel-refractory hormone refractory prostate cancer patients: randomized phase II study of ixabepilone or mitoxantrone and prednisone"

[1] Jonathan E. Rosenberg, [1] Vivian K. Weinberg, [2] W. Kevin Kelly, [3] Dror Michaelson, [4]Maha H. Hussain, [5] George Wilding, [6] Mitchell Gross, [1] Douglas Hutcheon, [1] Eric J. Small, MD

[1]University of California, San Francisco, [2]Memorial Sloan-Kettering Cancer Center, [3]Harvard Cancer Center, [4]University of Michigan, 5University of Wisconsin Comprehensive Cancer Center, [6]Cedars-Sinai Comprehensive Cancer Institute**.** Submitted, December 2006.

Conclusions:
We have demonstrated that the Vitatex technology can be used to isolate CTCs for genomic analysis. High quality DNA is able to be isolated from these cells. oCGH using CTC DNA isolated by the Vitatex system suggests that recurrent genomic alterations are present in CTCs, and are not present in white blood cells from these patients. Specimen collection continues in both chemotherapy-refractory and chemotherapy-naïve HRPC patients. Once sufficient numbers of specimens have been obtained, we will be able to begin to evaluate the genomic alterations associated with CTCs in HRPC in general, and chemotherapy resistance in particular. The Agilent array technology has a high resolution allowing the identification of specific genes that may be altered in metastatic and chemotherapy refractory HRPC.

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